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Altered Pattern of Cytokine Production by Peripheral Blood CD2+ Cells From B Chronic Lymphocytic Leukemia Patients

Eduardo Reyes,[1] Alfredo Prieto,[1] Flavio Carrión,[1] Julio García-Suarez,[2] Fatima Esquivel,[1] Cristina Guillén,[1] and Melchor Alvarez-Mon[1,3]*

[1]Departamento de Medicina, Universidad de Alcalá de Henares, Madrid, Spain
[2]Servicio de Hematología, Hospital Universitario Príncipe de Asturias, Spain
[3]Servicio de Medicina/Enfermedades del Sistema Inmune, Hospital Universitario Príncipe de Asturias, Spain

To determine if activation-induced cytokine production is altered in CD2+ lymphocytes from B-CLL patients, cytokine levels were determined by ELISA in supernatants of PHA-stimulated cultures of CD2+ cells from 33 B-CLL patients and 22 healthy controls. The production of Interferon γ (IFN-γ) and Tumor Necrosis Factor (TNF-α) by mitogen-activated CD2+ lymphocytes from B-CLL patients was higher than that found in healthy controls, while no differences were found in TNF-β production. IFN-γ and TNF-α levels determined at 72 h in PHA-stimulated CD2+ cell cultures from B-CLL patients statistically correlated with the percentages of CD3+CD45RO+ and CD3−CD56+ lymphocytes, respectively. Although there were differences in the production kinetics of interleukins (ILs) 2 and 4 between B-CLL patients and the healthy controls, no differences were found at the time when the levels of both interleukins peak. The production of both IFN-γ and IL-4 by PHA-stimulated CD2+ lymphocytes from non-smouldering B-CLL patients was significantly higher than that from smouldering B-CLL patients while no significant differences were found in the production of IL-2, TNF-α, and TNF-β between the two B-CLL patient groups. These data suggest that functional alterations in the production of cytokines by CD2+ cells from B-CLL patients could help to explain the expansion of leukemic cells in B-CLL patients. Am. J. Hematol. 57:93–100, 1998. © 1998 Wiley-Liss, Inc.

Key words: B chronic lymphocytic leukemia; smouldering B-CLL; interferon; tumor necrosis factor; interleukin

INTRODUCTION

B Chronic Lymphocytic Leukemia (B-CLL) is a common malignant lymphoproliferative syndrome of highly variable clinical course and is characterized by the clonal proliferation and accumulation of CD5+ B lymphocytes [1–5]. Although the neoplastic transformation involves B lymphocytes, phenotypic and functional alterations in the T lymphocyte and Natural Killer (NK) cell compartments have been described [6–12]. The pathogenic significance of these non-tumoral lymphocyte abnormalities in B-CLL patients remains elusive.

The phenotypical alterations found in the T cell compartment in B-CLL include the redistribution of the T cell subsets and the increased expression of activation antigens. A decreased CD4 to CD8 ratio [9,13–16] and a low intensity expression of these antigens has been described [17]. A drop in the CD45RA expression in the CD4 subset has also been reported [13,18,19]. An increased expression of the antigen activation markers CD25 and class II molecules of the HLA system in T lymphocytes from B-CLL patients has been also observed [13,20]. T cells from B-CLL patients also show

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E. Reyes and A. Prieto are joint first authors.

*Correspondence to: Melchor Alvarez-Mon, Departamento de Medicina, Universidad de Alcala de Henares, Carretera Madrid-Barcelona, km 33,600, Alcala de Henares, Madrid, Spain.

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functional alterations, decreased helper activity [9,21], and impaired proliferative response [7,9,22] to polyclonal stimulation with mitogens. These alterations in the T cell compartment of B-CLL patients have been related to the clinical progression of the disease [13,15,17,19]. It has been suggested that the impaired T cell function may be involved in the induction and/or maintenance of the leukemic B cell accumulation [23–25]. In this sense it has been shown that some T cell-derived cytokines can promote the growth and/or survival of B-CLL leukemic cells in vitro [23].

Several cytokines can regulate the proliferation and the programmed cell death of B-CLL leukemic cells [26–34]. IL-2, TNF-α, and TNF-β are known growth factors for B-CLL cells [27,27], whereas IL-4 and IL-6 antagonize the growth factor effect of IL-2 and TNF-α [28,29]. Transforming Growth Factor β (TGF-β) can also inhibit B-CLL cell growth [30]. IFN-γ [26], IL-2 [31], and IL-4 [32,33] inhibit whereas IL-10 induces apoptotic cell death of B-chronic lymphocytic leukemia cells [34]. The leukemic B cells produce some of these cytokines that regulate their own growth [27]. However, the relevance of these autocrine loops in the support of growth and viability of B leukemic cells remains uncertain since highly purified leukemic B cells die spontaneously by apoptosis in culture [26]. These data suggest that leukemic cells from B-CLL require T cell derived growth factors for survival and growth. Thus, the cytokines produced by T lymphocytes and NK cells might therefore be involved in the accumulation of leukemic cells in B-CLL patients.

Because peripheral blood mononuclear cells (PBMCs) from B-CLL patients have high percentages of tumoral B cells, enrichment for T and NK cells was required to compare their cytokine productions with those of the same cells in healthy controls. We have investigated the production of cytokines by PHA-stimulated purified CD2+ cells from B-CLL patients. We have selected for this study those cytokines produced by CD2+ cells that have known effects on the regulation of growth (IL-2, TNF-α, and TNF-β) or on the inhibition of programmed cell death (IL-2, IL-4, and IFN-γ) of B-CLL leukemic cells.

MATERIALS AND METHODS

Patients

Thirty-three patients (13 women, 20 men; mean age, 68 years: range 37 to 85) fulfilling the histopathologic, clinical, and immunologic criteria for B-CLL [35] were studied. None of the patients had received any treatment within the 3 last months prior to the study. The patients included in this study had no evidence of current acute or chronic disease other than B-CLL, nor a history of pathological conditions with possible effects on the immune system. The clinical stage was assessed according to the Binet system [36]. The criteria for smouldering B-CLL were also included [37]. Sixteen patients had smouldering B-CLL (lymphocyte count median, 14,000/μl, range 3,470 to 29,500), 17 patients had non-smouldering B-CLL (lymphocyte count median, 25,500/μl, range 5,400 to 222,700). In this group 10 patients had stage A, 6 patients had stage B, and the other patient corresponded to stage C. Twenty-two age- and sex-matched healthy controls were selected for the study. All patients and healthy controls gave their informed consent to the experimental protocol.

Cell Separation

PBMCs were obtained by centrifugation in Ficoll-Hypaque (Lymphoprep Nyegaard and Co., Oslo, Norway) density gradient. T cells were purified by double rosetting with sheep red blood cells pretreated with 2-aminoiethylisothio-uronium bromide, as previously described [22]. After counting, cells were resuspended in RPMI 1640 (Whitaker Bioproducts, Walkersville, supplemented with 10% heat-inactivated fetal bovine serum (Biochrom KG, Berlin, Germany), 2 mM L-glutamine (Biochrom KG), 25 mM Hepes (Biochrom KG), and 1% penicillin-streptomycin (Difco Lab, Detroit, MI). This will be referred to as complete medium (CM). Cell viability as checked by trypan blue exclusion was always greater than 95%. The purity of phenotypically defined CD2+ lymphocytes in the rosette cellular preparations was in all cases higher than 90%.

Measurement of Cytokine Productions

Supernatants were obtained by culturing T lymphocytes from normal controls and B-CLL patients at a density of 5×10^6 cells/ml in CM. Cultures were incubated in the presence or absence of 10 μg/ml phytohemagglutinin (PHA) (Difco Lab) and cell culture supernatants were harvested at 24 and/or 72 h of incubation, sterilized by filtration through a 0.22-μm filter (Millipore Company, Bedford, CA), aliquoted, and quickly stored at −70°C until measurement. Concentrations of cytokine were assayed using commercially available enzymoimmunoassay kits. IFN-γ, TNF-α, and IL-4 test kits were purchased from Genzyme Corporation, (Cambridge, MA), IL-2 test kit from T Cell Diagnostics (Cambridge, MA), and TNF-β test kit from R&D Systems (Minneapolis, MN). The results are expressed as pg/ml. The detection limit of the IFN-γ, TNF-α, IL-4, TNF-β, and IL-2 test kits are 100, 12, 45, 7, and 59 pg/ml, respectively.

Staining of Cells and FACS Analysis

The immunofluorescence studies were performed on fresh purified CD2+ lymphocyte fractions. For immunofluorescence staining, CD2+ lymphocytes were incu-

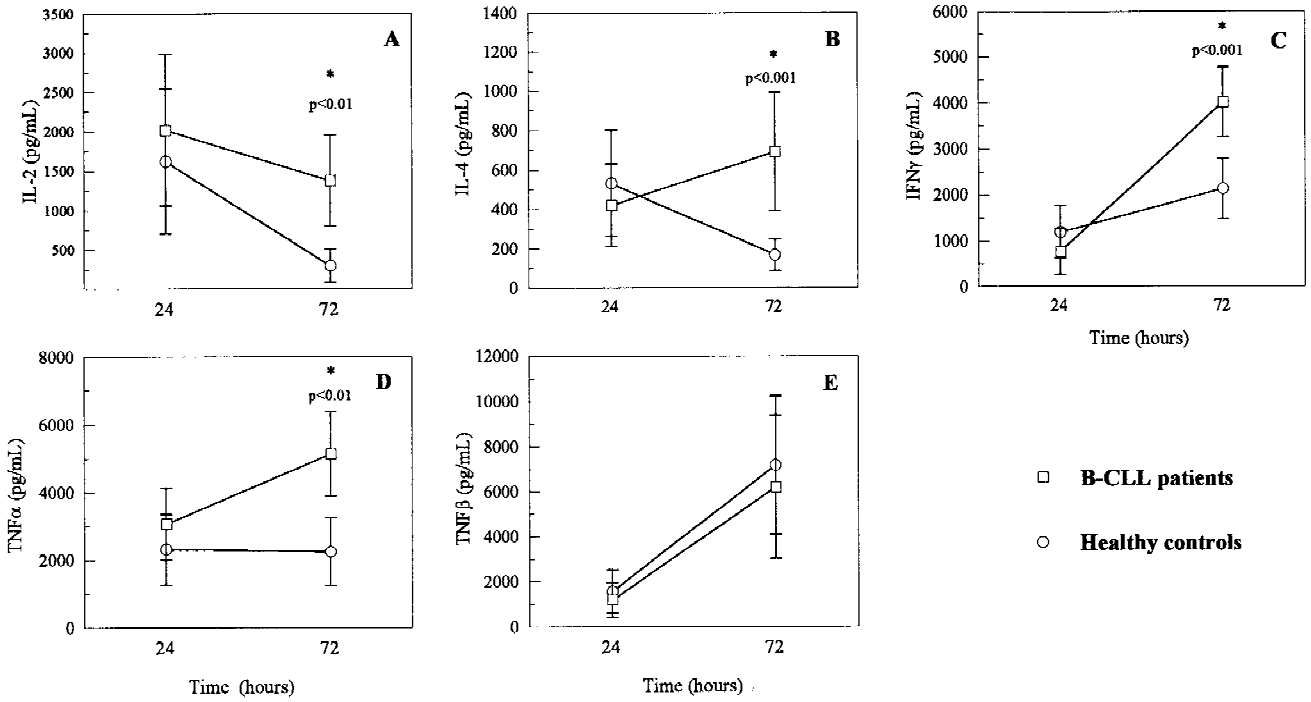

Fig. 1. Cytokine levels on supernatants from phytohemagglutinin (PHA)-stimulated CD2+ cell cultures. Interleukin (IL)-2 (A), IL-4 (B), IFN-γ (C), tumor necrosis factor (TNF)-α (D), and TNF-β (E) levels were measured on supernatants from PHA stimulated CD2+ cell cultures from 33 B chronic lymphocytic leukemia (B-CLL) patients and 22 healthy controls at 24 and 72 h of culture. Results are expressed as mean ± SD in pg/ml. *P* values were determined by the Student's *t*-test. *Significant differences between B-CLL patients and healthy controls. *P* values corresponding to differences between B-CLL patients and healthy controls are shown. *P* values corresponding to differences in the kinetic of each group are indicated in the text. No detectable levels of cytokines were detected in the supernatans of unstimulated cultures.

bated with combinations of fluorescein isothiocyanate (FITC, green), phycoerythrin (PE, orange), and Peridinin Chlorophyll Protein conjugate (PerCP, red)-labeled monoclonal antibodies (MoAbs). The MoAbs were used in three-color combinations to phenotypically define the enriched T cell fractions (FITC/PE/PerCP): anti-CD19 (all B cells)/anti-CD2 (all T cells and NK cells)/anti-CD3 (all T cells); anti-CD2/anti-CD56 (NK cells, some CTL)/anti-CD3, anti-CD4 (MHC class II restricted lymphocytes/anti-CD8 (MHC class I restricted lymphocytes)/anti-CD3 and anti-CD45RA (unprimed cells)/anti-CD45RO (memory cells)/anti-CD3.

Anti-CD45 FITC/anti-CD14 PE combination was used to determine residual monocytes in the purified CD2+ cell fractions. Control studies comprising unstained cells and cells incubated with isotype-matched irrelevant FITC-, PE-, and PerCP-labeled MoAbs were performed with each experiment. All MoAbs were obtained from Becton & Dickinson (Mountain View, CA). Acquisition and analysis for the three-color immunofluoresence procedures were carried out with a FACScan flow cytometer using Lysis II software (Becton Dickinson, San Jose, CA).

Statistical Analysis

Mean values were compared using a Student's *t* test. When data did not adopt normal distribution a nonparametric Mann-Whitney U test was used. *P* values were considered significant when they were less than 0.05. Correlations between groups were analyzed using Pearson's test. Analysis of data was done using StatView software 4.02 (Abacus Concepts, Inc., Berkeley, CA) on Macintosh Centris 610.

RESULTS

PHA-Induced IL-2 and IL-4 Productions by Purified CD2+ Lymphocytes From B-CLL Patients

The levels of cytokines produced by PHA-stimulated purified CD2+ cell fractions from B-CLL patients and healthy controls were studied after 24 and 72 h of culture. As can be seen in Figure 1A and B, the IL-2 and IL-4 concentrations measured in the culture supernatant of PHA-stimulated CD2+ cells from healthy controls at 24 h of culture were significantly higher than those measured after 72 h of culture ($P < 0.01$ and $P < 0.05$, respectively). However, the IL-2 and IL-4 concentrations

found in the cultures of PHA-stimulated CD2+ cells from B-CLL patients after 24 h were similar to those found at 72 h of culture. At 24 h of culture, no significant differences were found between the levels of IL-2 or IL-4 produced by PHA-stimulated CD2+ cells from B-CLL patients and healthy controls. However, at 72 h of culture, the concentrations of IL-2 and IL-4 in the same kind of cultures from B-CLL patients were significantly higher than in those cultures from healthy controls ($P < 0.01$ and $P < 0.001$, respectively).

PHA-Induced IFN-γ and TNF-α Productions by Purified CD2+ Lymphocytes From B-CLL Patients

As can be seen in Figure 1C and D, the IFN-γ and TNF-α levels measured in supernatants from PHA-stimulated CD2+ cell cultures from healthy controls at 24 h of culture were similar to those measured in the supernatant cultures at 72 h of culture. However, the IFN-γ and TNF-α concentrations measured in PHA-stimulated cultures from B-CLL patients were significantly lower at 24 h of culture than at 72 h of culture ($P < 0.001$ and $P < 0.05$, respectively). There were no significant differences in TNF-α and IFN-γ concentrations measured in supernatants of PHA-stimulated CD2+ cell cultures from B-CLL patients and controls at 24 h of culture. However, IFN-γ and TNF-α concentrations measured at 72 h in the PHA-stimulated cultures from B-CLL patients of culture were significantly higher than those found in cultures from healthy controls ($P < 0.001$ and $P < 0.01$, respectively).

TNF-β Production by Purified CD2+ Lymphocytes From B-CLL Patients

The TNF-β concentrations measured in the culture supernatants of PHA-stimulated CD2+ cells from both groups of subjects at 72 h of culture were higher than those measured at 24 h of culture ($P < 0.01$ controls; $P < 0.001$ B-CLL patients). No differences were found between B-CLL patients and healthy controls at 24 h nor at 72 h in the TNF-β concentrations measured in these PHA-stimulated cultures (Fig. 1E).

Very low or non-detectable concentrations of IL-2, IL-4, IFN-γ, TNF-α, and TNF-β were measured in the supernatants at 24 and 72 h of unstimulated cultures of purified $CD2^+$ cells from B-CLL patients and healthy controls.

Biological Correlations Between Lymphocyte Subpopulations and Cytokine Production

The composition of CD2+ cell fractures was studied in order to determine if differences in the levels of cytokines detected in culture supernatants from B-CLL patients and controls could be related to changes in the proportions of different subpopulations. The percentages of CD2+, CD8+, CD3+CD8+, CD3−CD8+, and CD19+ lymphocytes and of CD14+ monocytes in the CD2+ purified cellular fractions from B-CLL patients and healthy controls were similar (Table I). However, the percentages of the CD3+, CD4+, and CD3+CD45RA+ lymphocytes in B-CLL patients were lower while the percentages of the CD3−CD56+ and CD3+CD45RO+ lymphocytes were higher than those found in healthy controls (*P* values corresponding to differences between B-CLL patients and healthy controls are shown in the Table I).

TABLE I. Cell Subsets in Fresh Purified CD2+ Cell Fractions From Peripheral Blood in B-CLL Patients and Healthy Controls[†]

CD	B-CLL patients (n = 33)	Healthy controls (n = 22)
CD2+	95 ± 2	96 ± 2
CD3+	81 ± 8**	86 ± 2
CD4+	47 ± 11**	53 ± 9
CD8+	41 ± 10	38 ± 8
CD3+CD8+	33 ± 15	32 ± 11
CD3−CD8+	8 ± 6	6 ± 4
CD3−CD56+	14 ± 10*	10 ± 5
CD3+CD45RA+	33 ± 9***	55 ± 14
CD3+CD45RO+	50 ± 15***	33 ± 10
CD14+	2 ± 1	2 ± 1
CD19+	3 ± 2	2 ± 1

[†]Expression of surface antigens was determined in the fresh purified CD2+ lymphocyte fractions from B-CLL patients and healthy controls by flow cytometry. Results are expressed as mean ± SD. *P* values were determined by the Student's *t*-test. Number of patients and controls is shown in parentheses.

*$P < 0.05$ compared with healthy controls.

**$P < 0.01$ compared with healthy controls.

***$P < 0.01$ compared with healthy controls.

The existence of correlations between the percentages of the different lymphocyte subsets in CD2+ cell fractions from B-CLL patients and healthy controls and their PHA-induced lymphokine production was analyzed. As can be seen in Table II, the levels of IFN-γ at 72 h in PHA-stimulated cultures were statistically correlated with the percentages of the CD3+CD45RO+ T cells in the CD2+ cell fractions from B-CLL patients ($r = 0.53$, $P < 0.05$). The levels of TNF-α at 72 h of culture were statistically correlated with the percentages of the CD3−CD56+ NK cells in the CD2+ cell fractions from B-CLL patients ($r = 0.56$, $P < 0.05$). The PHA-induced IL-2 levels in CD2+ cell cultures from B-CLL patients after 72 h of culture were significantly and inversely correlated with the percentages of CD3−CD8+ cells ($r = -0.46$, $P < 0.05$). No significant correlation was found between the phenotypically defined lymphocyte subpopulations and the levels of TNF-β and IL-4 measured in supernatants of PHA-stimulated CD2+ cultures from B-CLL patients and healthy controls.

TABLE II. Correlations Between Lymphocyte Subpopulations in Fresh Purified CD2+ Cell Fractions and Concentrations of Cytokines Measured in Culture Supernatants of These PHA-Stimulated Cell Fractions at 72 H of Culture*

		B-CLL patients (n = 33)		Healthy controls (n = 22)	
Subpopulation	Cytokine	*P* value	r	*P* value	r
CD3+CD45RO+	IFN-γ	< 0.05	0.53	> 0.05	0.47
CD3–CD56+	TNF-α	< 0.05	0.56	> 0.05	0.26
CD3–CD8+	IL-2	< 0.05	−0.46	> 0.05	−0.34

*Correlations between the lymphocyte subpopulations in fresh purified CD2+ cell fractions from B-CLL patients and healthy controls and the culture levels of cytokines were determined using the Pearson's test.

TABLE III. IL-2, IL-4, IFN-γ, TNF-α, and TNF-β Concentrations Measured on Supernatants From CD2+ Purified Cell Fractions From Smouldering and Non-Smouldering B-CLL patients After 72 H Culture*

	B-CLL patients	
Cytokine	Smouldering B-CLL (n = 16)	Non-smouldering B-CLL (n = 17)
IL-2	1,957 ± 333	2,152 ± 749
IL-4	375 ± 101	1,022 ± 362 *P*<0.01**
IFN-γ	3,261 ± 475	4,762 ± 564 *P*<0.05**
TNF-α	5,650 ± 710	4,520 ± 1,140
TNF-β	5,438 ± 869	6,938 ± 1,097

*Purified CD2+ (5×10^6) lymphocytes/well were cultured in complete medium in the presence of PHA (10 μg/ml) and supernatants were harvested after 72 h of incubation. IL-2, IL-4, IFN-γ, TNF-α, and TNF-β levels were determined in the supernatants of CD2+ lymphocyte suspensions by enzymoimmunoassay. Results are expressed as mean ± SD in pg/mL. *P* values were determined by the Student's *t*-test.
**Indicates significant difference with smouldering B-CLL.

Cytokine Production by Purified CD2+ Cells From Smouldering B-CLL and Non-Smouldering B-CLL Patients

The cytokine production by PHA-stimulated purified CD2+ cells from B-CLL patients in different clinical stages of the disease was analyzed (Table III). The 33 patients were divided in two groups according to the criteria for smouldering B-CLL previously described by Monserrat et al. [37]. The first group included 16 patients with an early stage (smouldering B-CLL) and the second group 17 patients with more advanced disease (non-smouldering B-CLL). The levels of cytokines at 72 h of culture in both groups of patients are shown in Table III. The levels of IFN-γ and IL-4 produced by PHA-stimulated CD2+ lymphocytes from non-smouldering B-CLL patients were significantly higher than those found in smouldering B-CLL patients ($P < 0.05$ and $P < 0.01$, respectively). The levels of both IFN-γ and IL-4 produced by CD2+ cells from healthy controls were closer to those of smouldering B-CLL patients than to those found in non-smouldering B-CLL patients. No significant differences were found in the production of IL-2, TNF-α, and TNF-β by PHA-stimulated CD2+ lymphocytes at 72 h between the two groups of B-CLL patients. No statistical differences in cytokine levels were found between non-smouldering stage A patients and non-smouldering stage B patients (data not shown).

DISCUSSION

This study has found an abnormal cytokine secretion pattern in CD2+ cells from B-CLL patients after polyclonal stimulation. PHA activated CD2+ cells from B-CLL patients produced higher levels of IFN-γ and TNF-α those from healthy controls. However, TNF-β levels were similar in both groups. The residual concentration of IL-2 and IL-4 in the PHA-stimulated CD2+ preparations from B-CLL at 72 h of culture was significantly higher than that found in healthy controls. We have found that the increased IFN-γ production was statistically correlated with the increased percentage of CD45RO+ T cells in CD2+ cell fractions from B-CLL patients. The increased TNF-α production was also statistically correlated with the increased percentage of CD3−CD56+ NK cells in CD2+ cell fractions from B-CLL patients. These results suggest that the disordered T cell subsets and NK cells present in CD2+ fractions from B-CLL patients are responsible for the abnormal cytokine production patterns. We have also found that CD2+ cells from those patients with more advanced disease (non-smouldering B-CLL patients) produced larger amounts of IL-4 and IFN-γ than those with less advanced disease (smouldering B-CLL patients).

Autocrine production of growth factors by B-CLL leukemic cells has been proposed as a pathogenic mechanism in B-CLL [26,27,38–40]. However, the relevance of autocrine loops in the support of growth and viability of B leukemic cells remains uncertain since highly purified B-CLL cells die spontaneously by apoptosis in culture [26]. It has also been reported that T cell culture supernatants are required for growth and colony formation of B-CLL leukemic cells [41]. These data show that leukemic cells from B-CLL require T cell derived growth factors for survival and growth. Studies on mitogen-induced cytokine production by PBMCs from B-CLL patients have found strong correlations between percentages of T cells and the levels of some cytokines produced after polyclonal in vitro stimulation [24,25], indicating that T cells are important producers of cytokines in PBMCs from B-CLL patients. It has been reported that the production of cytokines by PBMCs from B-CLL patients is different from that found in cells from healthy controls [24,25]. Since the main population of PBMCs in these patients in the leukemic B cell population, the al-

tered production of cytokines can be partially ascribed to a reduced percentage of CD2+ cells in PBMCs from B-CLL patients. For this reason, we investigated the activation-induced production of cytokines in purified CD2+ cells.

It has been described that T lymphocytes and NK cells from B-CLL patients show abnormal functional behaviors in vitro [7,9,21,22]. These altered lymphocyte functions might be involved in the abnormal pattern of cytokine production reported in this paper. It is well documented that the expression of the lymphocyte genes by T lymphocytes and NK cells is related to their functional stage [42,43]. Heterogeneous patterns of lymphokine expression have also been found in the different subsets of these lymphocyte populations [43]. Thus, the altered distribution of the T lymphocyte and NK cell subsets found in B-CLL patients may be involved in their abnormal cytokine production. We have found a significant correlation between the percentage of CD3+CD45RO+ T cells in the CD2+ cells from B-CLL patients and their IFN-γ production. Furthermore, the enhanced IFN-γ production by CD2+ cell fractions from B-CLL patients, is associated with their increased percentage of CD3+CD45RO+ T lymphocytes that is higher than that found in healthy controls. It has been reported that CD45RO+ T lymphocytes from healthy donors produce more IFN-γ than their CD45RA+ counterparts [43,44]. These findings suggest that the increased IFN-γ production by PHA-activated CD2+ lymphocytes from B-CLL patients may be related to the increased degree of activation of the T lymphocytes and could be caused by expansion of CD45RO+ T cells. Selective expansion of T lymphocyte clones with antigenic reactivity against autologous leukemic B-CLL cells has been reported in B-CLL patients [45]. We have found that T lymphocytes from B-CLL patients present increased proportions of phenotypically defined (CD45RO+) more differentiated T cells. These results suggest that, as a result of immune activation, a high proportion of T lymphocytes from B-CLL patients have been primed in vivo. These primed T lymphocytes, which includes both effector T cells and memory cells, as a result of their maturation could secrete larger amounts of IFN-γ than their naive counterparts.

The pattern of cytokine secretion found in CD2+ cells from B-CLL patients is not related to a clear Th1 or Th2 preference because they both produce increased IL-4 and IFN-γ, but similar TNF-β levels. Our data show that T lymphocytes from B-CLL patients have increased percentages of more differentiated T cells and suggest that CD2+ cells from B-CLL patients produce increased quantities of both Th1 and Th2 lymphokines but we cannot conclude whether individual cells are producing both Th1 and Th2 cytokines or not. It has been recently reported that accessory cells could have a role in the cytokine production by CD4+ cells in B-CLL [46]. Accessory B cells induce a Th2 pattern of cytokine production whereas monocytes tend to induce a Th1 pattern [46]. However, we did not find statistically significant differences in these accessory cell populations between CD2+ cell fractions from B-CLL patients and healthy controls. Thus, our results cannot be explained by differences in the proportion of these accessory cells in the purified CD2+ cell fractions.

The abnormal pattern of cytokine secretion observed in CD2+ lymphocytes from B-CLL patients could have several pathogenic meanings. It has been reported that IFN-γ inhibits apoptotic cell death and promotes survival of leukemic B-CLL cells in vitro [26]. This effect may also be important in vivo, since IFN-γ may extend the life span of the malignant cells and contribute to their accumulation in the organism [26]. B-CLL patients have been reported to have increased serum levels of IFN-γ [26]. The enhanced in vitro production of this cytokine by CD2+ cells from B-CLL patients reported in this article might contribute to our understanding of the previously reported increased serum levels of this cytokine in these patients [26]. The different IFN-γ production by CD2+ cells from B-CLL patients in different stages of the disease suggests the possible pathogenic relevance of this cytokine. Of interest is that IFN-γ production by CD2+ cells from B-CLL patients in early stages of the disease is significantly lower than that of CD2+ cells from patients with more advanced disease. It has been described that TNF-α is a growth factor for leukemic cells from B-CLL patients [27]. Our data also demonstrate that PHA-stimulated CD2+ cells from B-CLL patients have an increased capacity to produce TNF-α. Taken together, these results suggest that the increased production of these cytokines by CD2+ cells could contribute to the expansion of leukemic cells in B-CLL patients.

IL-2 production by enriched T lymphocyte cell fractions from B-CLL patients has been previously reported as normal [22,47] or decreased [48]. We have found that both IL-2 and IL-4 production by PHA-stimulated CD2+ cells from B-CLL patients are normal after 24 h of culture. Apparent discrepancies between our results of IL-2 production and those of Kay and Kaplan [48] may be due to differences in the method for CD2+ cell purification. In our study, all CD2+ cell fractions were obtained after two consecutive rossetting procedures. The study of Kay and Kaplan that reported lower IL-2 production in B-CLL patients was done with CD2+ purified cell fractions that were obtained after only one rossetting procedure [48].

Of interest is that after 72 h of culture the residual levels of both cytokines IL-2 and IL-4 found in the supernatants of PHA-stimulated CD2+ cells from B-CLL patients are significantly higher than those measured in

healthy controls in similar experimental conditions. Different potential explanations of these results may be proposed. It is possible to consider that the production of both IL-2 and IL-4 is normal but their usage is defective in CD2+ cells from B-CLL patients. It has also been described that although the IL-2 production in CD2+ cells from B-CLL patients is apparently normal, their proliferative response to PHA is reduced even when exogenous IL-2 is added to the culture medium [25]. This lack of proliferation despite normal IL-2 production gives further support to the concept of a defective use of IL-2 in these CD2+ cells from B-CLL patients. However, an increased production of these cytokines by the activated CD2+ cells from B-CLL patients cannot be excluded and might be involved in the differences found between B-CLL patients and healthy controls. Since IL-2 and IL-4 do have an effect on viability and proliferation of leukemic B cells from B-CLL patients in different experimental systems [31–33], it seems sensible to think that the abnormal behavior of production and/or usage of both cytokines by CD2+/cells from B-CLL patients may provoke an increased exposure of leukemic cells to these molecules, which could contribute to maintain their viability.

It is known that the activation of T lymphocyte and NK cells occurs in vivo and is dependent on the exogenous and endogenous antigenic stimulation such as that given by leukemic B cells [45]. This antigenic stimulation varies along time and is mainly localized in certain anatomical compartments. Hence the potential pathogenic signification of the abnormal pattern of cytokine production by CD2+ cells might be relevant in specific micro-environments where T lymphocyte are abundant and where their activation plays a relevant physiological role such as in T cell areas of lymph nodes. These micro-environments are usually infiltrated by the leukemic B-CLL cells. Hence, is in these micro-environments were the leukemic B-CLL cells could be exposed to the cytokines produced by CD2+ cells undergoing activation; and is in these micro-environments where it has been demonstrated that the apparently slow-dividing monoclonal B-CLL cells do proliferate actively [49]. It seems sensible that cytokines produced by T cells could play a crucial role in the regulation of proliferation and survival of B-CLL cells in vivo like it has been demonstrated in vitro [41,50].

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